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Biochimica et Biophysica Acta 1317 (1996) 149–154

BBA Biochimica et Biophysica Acta

A conformational epitope in the N-terminus of the *Escherichia coli* heat-stable enterotoxins is involved in receptor-ligand interactions

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Received 9 July 1996; accepted 18 July 1996

Abstract

The heat-stable enterotoxins are a family of low molecular weight, cysteine rich peptide toxins which are one of the major causes of watery diarrhea in children and adults. These toxins bind to a cell surface receptor in intestinal cells and mediate their action through elevation of intracellular cyclic GMP. We have generated a monoclonal antibody to these peptide toxins which is able to neutralise the activity of the peptides in a human colonic cell line, the T84 cell line. The monoclonal antibody, ST:G8, appears to be directed to an epitope distinct from antibodies previously generated, and prior incubation of this antibody, or Fab generated from this antibody, with full length STh and STp peptides prevents cGMP accumulation in T84 cells. This inhibition is a direct result of the antibody preventing binding of the peptides to the receptor. ST:G8 Mab does not recognize a 13-mer biologically active analog of STp, comprising the core sequence of STp peptide, suggesting that it is directed to a region in the N-terminus of the peptides, which may modulate receptor interaction/activation. The antibody recognizes a conformational epitope in the ST peptides, since reduction and carboxyamidation of ST abolishes antibody cross-reactivity. Differential cross-reactivity of the Mab with STh and STp peptides, which differ markedly only in their N-termini, suggests that this antibody recognizes a distinct conformation in the two peptides, which is essential for receptor interaction.

Keywords: Heat-stable enterotoxin; Human ST receptor; T84 cell; Monoclonal antibody

1. Introduction

The heat-stable enterotoxins (ST) are a family of low molecular weight peptides produced by a variety of pathogenic bacteria, and are one of the major causes of watery diarrhea the world over [1,2]. These toxins are characterised by the presence of three disulphide bridges which are essential for full biological activity [3], and ST peptides produced by different pathogenic bacteria differ in the amino acids between the cysteine residues and in sequences towards the N-terminus of the central cysteine rich core [4–6] (Table 1). In fact, a 13-mer sequence containing the 6 cysteine residues in the toxin has biological activity in the suckling mouse assay [7,8], which is the model system usually used for in vivo assays for the ST peptides.

The ST peptides bind to a receptor present in intestinal cells. Cloning and biochemical characterisation of the receptor from both rat and human tissue indicates that it is a member of the family of membrane bound guanylyl cyclases [9]. Membrane-bound guanylyl cyclases serve as receptors for a number of different peptide ligands [10], and ligand binding to the extracellular domain of these receptors activates the cytoplasmic guanylyl cyclase domain, resulting in elevated levels of cGMP within the cells. In the case of ST peptides, the events involved subsequent to cGMP production are poorly defined, but probably occur by the cross-activation of protein kinase A by cGMP, and increased chloride secretion through the cystic fibrosis transmembrane regulator [11].

We have been interested in studying the interaction of ST peptides with the human receptor present in a human colonic cell line, the T84 cell line [12]. This cell line

Abbreviations: BSA, bovine serum albumin; CCEL, 13-mer peptide corresponding to core sequence of STp; DTT, dithiothreitol; ELISA, enzyme linked immunosorbent assay; Mab, monoclonal antibody; PBS, phosphate buffered saline; ST, heat-stable enterotoxin; STh, ST peptide of the human variety; STp, ST peptide of the porcine variety; STY72F, ST peptide with C-terminal tyrosine replaced by phenylalanine

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0925-4439/96/$15.00 Published by Elsevier Science B.V.
PII S0925-4439(96)00047-6

Table 1
Amino acid sequences of peptides used in this study

Peptide	Sequence	Species
STh	NSSNY**CC**EL**CC**NPA**C**TG**CC**Y	*E. coli*
STp	NTFY**CC**EL**CC**NPA**C**AG**CC**Y	*E. coli*
STY72F	NSSNY**CC**EL**CC**NPA**C**TG**CC**F	*E. coli*
CCEL	**CC**EL**CC**NPA**C**AG**CC**	*E. coli*
Yersinia ST	SDWD**CC**DV**CC**NPA**C**AG**CC**	*Yersinia enterocolitica*

mimics the normal human intestine in showing vectorially directed ion transport, and harbours the receptor for the ST peptides [13]. We have shown that a variety of ST peptides produced by a number of pathogenic bacteria, are able to elicit elevated levels of cGMP within T84 cells, presumably by interacting with the receptor present in these cells [14]. We have characterised this receptor in T84 cells, and partial purification indicated that it too is a member of the membrane associated form of guanylyl cyclase [15].

Monoclonal antibodies (MAb) serve as useful tools to delineate regions of a ligand which play an important role in interacting with the receptor. A number of monoclonal antibodies have been raised to ST peptides, some of them sequence- and some of them conformational-specific [16–18]. In a few cases, these antibodies are neutralising, either in the suckling mouse assay or in inhibiting cGMP production in T84 cells [17,18]. Interestingly, most of these antibodies have been mapped to the C-terminus core of the ST peptides, which is sufficient for full biological activity. In one case, a single residue at the N-terminus of STh, outside the core sequence, was essential for recognition of the antibody [18].

We have earlier suggested that apart from the C-terminus core of the ST peptides, sequences extended at the N-terminus may also modulate ST activity [14]. We have based this hypothesis on the fact that peptides which differ only marginally in the C-terminus core sequence have markedly different activities in a biological assay [14]. In this report, we show that a neutralising Mab which could be directed to the N-terminus of ST peptides is able to inhibit ST interaction with the receptor in T84 cells.

2. Materials and methods

2.1. Purification of ST peptides

STh and STY72F peptides were purified from hyperexpressing strains as detailed earlier [15,19]. Purity of the peptides was checked by reverse phase high performance liquid chromatography and peptides quantitated by amino acid analysis as described [20]. *Yersinia* ST and the 13-mer peptide corresponding to the core sequence of STp (CCEL) were synthesised and air oxidised to obtain biologically active peptides as described earlier [14]. STp was procured from Sigma (Mo., USA), and peptide quantitated after reconstitution. Bioactivity was monitored by elevation of intracellular cGMP levels on application of the peptides to monolayer cultures of T84 cells.

2.2. Culture and maintenance of T84 cells

T84 cells were procured from ATCC (CCL-248) and cultured as described earlier [14]. Following application of ST to monolayer cultures of T84 cells, intracellular levels of cGMP were monitored 15-30 min later by lysis of the cells in 0.1 M citric acid [14]. Radioimmunoassay was performed using ^{125}I-labelled succinyl tyrosyl methyl ester of cGMP. Under these conditions, less than 10% of cGMP produced was secreted into the culture supernatant [14].

2.3. Generation and purification of Mab ST:G8

Purified STY72F peptide was conjugated to bovine serum albumin using glutaraldehyde and BALB/c mice were immunised with 50 μg of the conjugate in complete Freund's adjuvant, intradermally. Fourteen days following primary injection, mice were boosted with the same amount of conjugate in incomplete adjuvant. One week following the booster, the mice were bled through the tail vein and serum titres of antibodies to ST peptides were monitored by ELISA. Mice which had significant levels of circulating antibodies were boosted with daily i.p. injections of conjugate (10 μg in PBS) for four days prior to fusion. Fusion was carried out by established protocols [12], and culture supernatants were tested by ELISA against ST coated plates (see below). The clone ST:G8 was established following two rounds of subcloning, and cells used for the generation of ascites fluid. IgG from the ascites fluid was purified by Protein A agarose (Pierce Chemical Company, Rockford, USA) and tested for subclass and light chain specificity by using subclass and isotype specific antisera (Sigma, St. Louis, MO, USA).

Fab fragments of the IgG was prepared by digestion of the purified IgG by papain. Briefly, 500 μg of purified IgG was incubated in 100 mM sodium acetate buffer, pH 5.5 containing 10 mM 2-mercaptoethanol, 1 mM EDTA and 5 μg of papain dissolved in acetate buffer. Digestion was continued for 6 h at 37°C in vacuo and quenched by the addition of 150 mM iodoacetamide. The mixture was dialysed against PBS, checked for digestion by SDS gel electrophoresis [21] and the mixture was added to 100 μl of Protein A agarose. Mixing was continued for 2 h at 25°C to remove Fc and any undigested IgG. The unbound fraction was then checked by SDS gel electrophoresis, and protein quantitated by the Bradford method [22].

2.4. ELISA procedure for ST peptides

The ST peptides were dissolved at concentrations of 2 μg/ml in 10 mM sodium phosphate buffer, pH 7.2,

containing 0.9% (w/v) sodium chloride (PBS) and 100 μl aliquots were added to ELISA plates (high binding, NUNC). Peptide was allowed to adsorb to the plates at 4°C for 16 h. Plates were washed, blocked with PBS containing 0.2% (w/v) bovine serum albumin (BSA), and antibody added suitably diluted in PBS/BSA/0.1% (v/v) Tween 20 (usually 1:1000 ascites fluid) and allowed to interact with the peptide for 1 h at 25°C. The bound antibody was monitored using a horseradish peroxidase-anti-mouse IgG conjugate (Bangalore Genei, India), and colour developed using 3,3′,5,5′-tetramethylbenzidine as a substrate.

2.5. Reduction and carboxyamidation of ST peptides

Peptides (10^{-5} M) were incubated in 100 mM Tris-HCl buffer, pH 8.8, containing 1 mM DTT, at 37°C, for 2 h. At the end of this time, iodoacetamide (5 mM) was added and incubation continued for a further 30 min in the dark at 25°C. The peptides were then used directly in a radioimmunoassay to monitor inhibition of ^{125}I-labelled STY72F (approx. 50 000 cpm) binding to the suitably diluted antibody. Controls included incubations performed in the presence of equivalent concentrations of native ST peptides.

2.6. Radioimmunoassay using radiolabelled STY72F and Mab

STY72F peptide was iodinated as described earlier and purified by reverse phase HPLC [15]. Suitable dilutions of ST:G8 were incubated with approx. 20 000–50 000 cpm of radiolabelled peptide in PBS containing 0.2% (w/v) BSA for 1 h at 37°C, in a total volume of 300 μl. Included during the assay were varying concentrations of unlabelled STh, STp and STY72F peptide. Incubation was continued for 1 h following which 1 ml of a chilled 0.2% (w/v) charcoal suspension (Norit A, Gibco) in PBS/BSA was added, and incubation continued for 5 min at 25°C. The samples were then centrifuged, supernatant discarded and charcoal bound radioactivity, which represented ST peptide unbound to antibody, was monitored.

2.7. Neutralisation of ST activity in T84 cells

ST peptides, (10^{-8} M), were incubated with 15 μg of purified IgG or varying concentrations of Fab fragment prepared from the purified IgG fraction, for 1 h at 37°C. in DMEM:F12 (Gibco) containing 0.2% BSA. At the end of this time, the solution was applied to T84 cells, which had been washed with DMEM:F12 medium. The cells were incubated for 30 min at 37°C, in an atmosphere of 5% CO_2, following which cells were washed and lysed in 400 μl 0.1 M citric acid. Samples of the cell lysate were assayed for cGMP production by radioimmunoassay as detailed earlier.

To monitor the inhibition of radiolabelled STY72F binding to the receptor present in T84 cells, membranes were prepared from T84 cells as described earlier [15]. Radiolabelled STY72F, (10^{-10} M) was incubated with the ascitic fluid (1:100 dilution), in 50 mM Hepes, pH 7.2, 4 mM $MgCl_2$, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 0.2% BSA in a total volume of 100 μl for 1 h at 37°C, and then added to 50 μg of T84 membrane protein, either in the presence or absence of unlabelled STh (10^{-7} M) under conditions described in detail earlier [15]. Following incubation for 1 h at 37°C, the samples were filtered through glass fibre filters (GF/B, Whatman) and counted.

3. Results

3.1. Production and characterisation of the Mab ST:G8

The Mab was raised to the STY72F peptide, which we have characterised earlier as a potent ligand for the human ST receptor present in T84 cells [15]. The particular clone that was identified was initially selected by its ability to interact with STh and STY72F peptides coated to the ELISA plate. The Mab ST:G8 was of the IgG2a subclass with κ light chain. Fig. 1 shows the reaction of the peptide to various purified and synthetic peptides corresponding to different ST peptides produced by *E. coli* and *Yersinia enterocolitica*. ST:G8 was able to recognize full length peptides produced by *E. coli* but did not react with the synthetic core sequence corresponding to a 13-mer peptide from the STp peptide. This suggested that the Mab was directed to an epitope not present in the 13-mer biologically active peptide, but required residues towards the N-terminus of the full length ST peptide. The Mab was specific to the peptides produced by *E. coli* and did not interact with the *Yersinia* ST (Fig. 1).

3.2. Neutralising effect of the Mab

In order to test the ability of ST:G8 to inhibit the bioactivity of ST peptides, we have monitored the production of cGMP in T84 human colonic cells, on application of ST peptides previously incubated with the antibody.

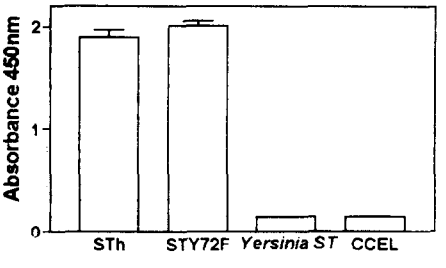

Fig. 1. Interaction of ST:G8 Mab with ST peptides. ST peptides were immobilised on ELISA plates and ST:G8 ascites fluid at a dilution of 1:50 000 was added to the plates. Following incubation for 1 h, bound antibody was detected using an anti-mouse IgG-horse radish peroxidase conjugate. Values represent the mean (±S.D.) of duplicate or triplicate determinations, with each experiment repeated thrice.

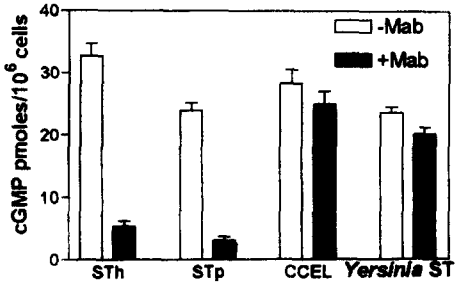

Fig. 2. Neutralisation of ST activity in T84 cells by ST:G8. Peptides (10^{-8} M) were incubated with purified IgG ST:G8 Mab as detailed in Section 2, and then applied to T84 cells in culture. cGMP produced was monitored by radioimmunoassay. Values represent the mean (±S.D.) of duplicate incubations, with each experiment repeated thrice.

Fig. 2 shows that in agreement with the data from the ELISA results, the antibody was able to neutralise the activity of *E. coli* STh and STp peptides, but not the activity of the 13-mer core sequence of STp or *Yersinia* ST. In order to determine whether this neutralisation was due to inhibition of peptide binding to the receptor, or in preventing a conformational change induced on ligand binding to the receptor present in T84 cells, we checked the ability of ST:G8 to inhibit the binding of labelled STY72F to the receptor in T84 cells. We incubated excess Mab ST:G8 with radiolabelled STY72F and then added it to membranes prepared from T84 cells, either in the presence or absence of unlabelled STh peptide. As can be seen from Fig. 3, the antibody was able to prevent the binding of radiolabelled ST peptide to its receptor, as efficiently as a large excess of unlabelled STh peptide. This clearly indicates that binding of the antibody to STY72F prevents its further interaction with the receptor, therefore inhibiting cGMP accumulation in the T84 cells, as shown in Fig. 2.

It is likely that there could be a steric constraint in allowing ST interaction with the receptor following antibody binding, perhaps contributed by the Fc fragment of the whole antibody molecule. We therefore prepared the

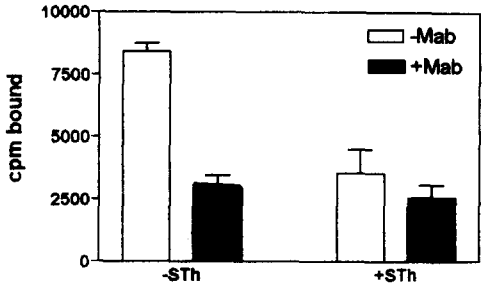

Fig. 3. Monoclonal antibody ST:G8 inhibits binding of STY72F to the ST receptor in T84 cells. ST:G8 Mab was incubated with ^{125}I-labelled STY72F, and the mixture added to T84 membranes, either in the presence or absence of a large excess of STh peptide. Receptor bound radioactivity was monitored following filtration through glass fibre filters. Values represent the mean (±S.D.) of duplicate determinations, with the experiment repeated thrice.

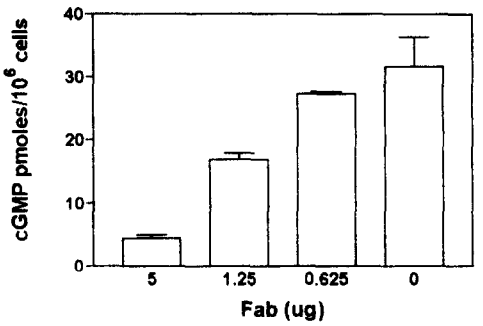

Fig. 4. Neutralisation of STh activity by Fab fragment prepared from purified ST:G8 Mab. Fab fragment was prepared from purified ST:G8 IgG, and incubated at indicated concentrations with STh (10^{-8} M) prior to addition to T84 cells. cGMP produced was monitored by radioimmunoassay. Values represent the mean (±S.D.) of duplicate determinations, with the experiment repeated thrice.

Fab fragment from purified whole Mab and the data in Fig. 4 show that the Fab fragment is also able to inhibit cGMP production on addition of ST to the T84 monolayer. While this still does not rule out the possibility of steric hindrance preventing ST bound to antibody from interacting with the receptor, it does suggest that the residues which may modulate receptor interaction are blocked following incubation with the antibody. It is pertinent to note that some antibodies to STh have been shown to be poorly neutralisable or even non-neutralising [14,18], suggesting that antibody interaction alone with the peptide may not prevent interaction of the peptide with the receptor.

In order to determine whether the antibody is directed to a sequential epitope or a conformational epitope, we reduced and carboxyamidated ST peptides and determined their reactivity with the antibody in a liquid phase radioimmunoassay. STh and STY72F peptides were reduced with DTT and carboxyamidated with iodoacetamide. The data shown in Fig. 5 indicates that reduced peptide were unable to inhibit the binding of labelled STY72F to the antibody, confirming that the antibody recognizes a conformation

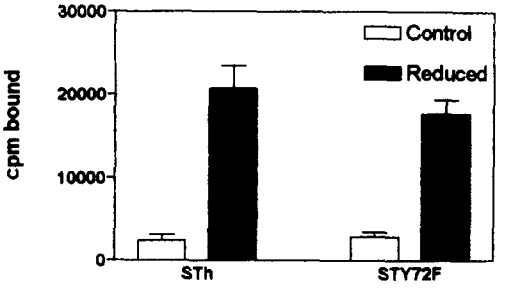

Fig. 5. Monoclonal antibody ST:G8 is directed to a conformational epitope in ST peptides. STh and STY72F peptides were reduced and carboxyamidated and used in radioimmunoassay to monitor inhibition of binding of native ^{125}I-labelled STY72F to ST:G8 Mab. Untreated peptide was also incubated in a similar manner and radioactivity associated with the antibody monitored following charcoal treatment. Values represent the mean (±S.D.) of duplicate determinations with each experiment repeated at least thrice.

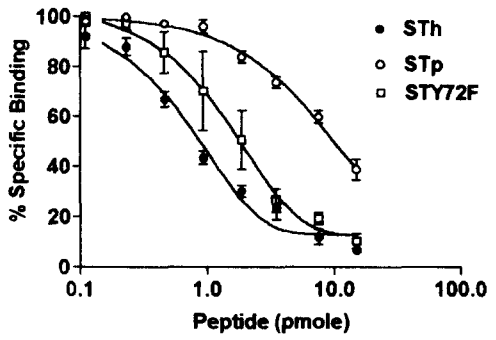

Fig. 6. Inhibition of binding of labelled STY72F to ST:G8 by ST peptides. Indicated concentrations of STh, STp or STY72F were incubated along with radiolabelled STY72F and purified ST:G8 IgG (0.5 μg/ml final dilution). Binding to antibody was monitored following charcoal addition. Values represent the mean ± S.D. of duplicate or triplicate determinations with experiments performed at least thrice.

presumably in the N-terminus of the ST peptide, which is destroyed on reduction and carboxyamidation.

The data presented here suggest that the Mab ST:G8 reacts with a conformational epitope present outside the core sequence of the ST peptides, and could be directed to an epitope composed of the N-terminal sequences along with the core sequence. We have shown here that the Mab recognizes STh, STY72F and STp sequences, the latter differing in sequence at its N-terminus (Table 1). We monitored the inhibition of binding of labelled STY72F to the antibody brought about by varying concentrations of STh, STp and STY72F. The data shown in Fig. 6 indicate that the concentration of STp required to inhibit labelled STY72F binding to the receptor is nearly 10-fold higher than STh, strongly suggesting that antibody recognition prefers a conformation contributed by the N-terminus of STh, rather then STp. It is interesting to note that all the 3 peptides used in this assay have a residue following the 13-mer core sequence (Y or F; Table 1) indicating that the C-terminal residue following the core sequence, may not contribute significantly to the epitope involved in receptor recognition.

4. Discussion

In this report, we describe the development of a Mab to the family of ST peptides produced by *E. coli*. We have shown that the antibody is able to inhibit the binding of the peptide to the human receptor and therefore neutralise cGMP production on ST application in T84 cells. We have demonstrated that this antibody is conformational specific and tried to address the problem of steric hindrance of antibody-ligand-receptor binding by showing that the Fab fragment prepared from the monoclonal antibody, is able to inhibit binding as well.

A number of groups have raised monoclonal antibodies to the ST peptides, and some have suggested their application for diagnostic purposes [17,23]. In all cases, as indeed in ours, the antibodies raised to the *E. coli* peptides do not interact with the peptides produced by *Yersinia* or non-01 *Vibrio* [17,18]. This clearly indicates that there are unique sequences in the latter peptides that modulate their interaction with the receptor, and which differ markedly in the *E. coli* and *Yersinia* peptides. It is therefore interesting to note that a small peptide such as ST may harbour more than one conformational epitope in its stretch.

The amino acid sequences which are essential for interaction with various monoclonal antibodies to STh have been determined by Takeda et al. [18]. A tyrosine at the C-terminus of STh was critical for one Mab interaction, and an epitope to another distinct Mab was suggested to be present towards the N-terminus of ST peptides. We feel that ST:G8 defines an epitope different to studies detailed earlier in that there does not appear to be a selectivity for the presence of a tyrosine at the C-terminal residue, since ST:G8 is able to interact with STY72F peptide with comparable affinity to STh. We also have evidence that the antibody is able to recognize a mutant STh peptide where the 5th amino acid from the N-terminus, tyrosine, has been replaced by tryptophan (data not shown). We therefore feel that this antibody recognizes a conformation at the N-terminus, generated by the correctly folded core sequence, which could be important in ligand-receptor interactions.

The crystal structure has been determined for an analog of the core sequence of the ST peptides, and defines three distinct conformations in the peptide [24]. However, no information is available to date as to a possible structure adopted by the N-terminal sequences of the peptides, which, as we have shown in this study, directly affects ligand binding to the receptor. It is conceivable that binding of an antibody to the N-terminus of the ST peptide could distort the final conformation of the C-terminus of the ST peptide and therefore prevent receptor interaction. Such information can only be obtained from the crystal structure of this antibody along with ST peptides.

It has been suggested that the 13-mer core sequence of the ST peptides is sufficient for full biological activity. However, no precise measurements have been made of the affinity of the 13-mer peptide to the receptor, nor a comparison with the native full length STp. It has been difficult to obtain a potent antiserum to this region, probably due to the similarity in structure of this region with a naturally occurring peptide, guanylin, which has been shown to interact with the same receptor as ST in T84 cells [25]. We would like to determine the epitope to which ST:G8 is directed by identifying the conformation adopted by ST in its interaction with ST:G8. This epitope could be identified by the use of constrained phage display libraries [26], and the sequence thus identified used for the generation of neutralising antibodies. It would be interesting to determine if such a sequence could inhibit or modulate ST interaction with the receptor. Such studies are underway in the laboratory.

Acknowledgements

We would like to acknowledge the help and useful discussions with Prof. Ann-Mari Svennerholm and Prof. Jan Holmgren of the University of Goteborg, Sweden. We also acknowledge the help of Animesh Nandi, Nivedita Roy and Manjiri Bakre for the culture of T84 cells and radioiodination of ST peptides. Financial assistance was obtained from the Rajiv Gandhi Foundation, New Delhi, India. BMG is a project assistant supported by the Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India.

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